

Title	Fluorescence diagnosis of metastatic lymph nodes using 5-aminolevulinic acid (5-ALA) in a mouse model of colon cancer.
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Citation	Journal of surgical research (2012), 176(2): 430-436
Issue Date	2012-08
URL	http://hdl.handle.net/2433/160047
Right	© 2012 Elsevier Inc.
Type	Journal Article
Textversion	author

Title Page

TITLE

Fluorescence diagnosis of metastatic lymph nodes using 5-aminolevulinic acid (5-ALA)
in a mouse model of colon cancer

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RUNNING TITLE

Fluorescence diagnosis of lymph node metastasis

CATEGORY

Gastrointestinal

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Abstract

Background: Lymph node metastasis is one of the most critical prognostic factors in patients with colorectal cancer. Although regional lymph nodes should be surgically resected and pathologically examined, techniques for the intraoperative diagnosis of lymph node metastasis remain to be well established. Fluorescence diagnosis using 5-aminolevulinic acid (5-ALA) is a promising technique for evaluating various malignancies. After exogenous administration of 5-ALA, protoporphyrin IX (PPIX) accumulates in malignant cells and can be detected as red fluorescence. In this study, we investigated the usefulness of fluorescence diagnosis using 5-ALA for the detection of lymph node metastasis in a mouse model of colon cancer.

Materials and Methods: An orthotopic colon cancer model was prepared by inoculating the cecal wall of nude mice with HCA7, a human colon adenocarcinoma cell line. After 3 weeks, 40 mg/kg of 5-ALA was administered intraperitoneally (IP) or orally (PO). Fluorescence diagnosis with a D-Light System (Karl Storz) was then performed after 3 or 6 hours.

Results: In the IP group, PPIX fluorescence was detected in metastatic lymph nodes as well as in other malignant lesions, including primary tumors and abdominal implantations, while non-metastatic nodes were fluorescence-negative. In contrast, no obvious fluorescence was detected in cancerous tissues in the PO group.

Conclusions: PPIX fluorescence induced by intraperitoneal injection of 5-ALA allows metastatic lymph nodes to be accurately diagnosed in this mouse model. This technique may facilitate the intraoperative diagnosis of lymph node metastases from colon cancer in a clinical setting.

Key Words: 5-aminolevulinic acid, lymph node, metastasis, fluorescence diagnosis, colon cancer.

Introduction

Colorectal cancer is a very common type of cancer worldwide and is now the third leading cause of cancer-related death in Japan [1, 2]. The presence of lymph node metastasis is clearly related to the prognoses of patients, and accurate diagnosis of nodal involvement is essential to planning therapeutic strategies, such as an extent of lymph node dissection and adjuvant chemotherapy [3-6]. The goal of surgical treatment for colon cancer is the resection of the involved bowel segment and its lymphatic drainage at the level of the primary feeding arterial vessel (e.g., ileocolic, middle colic, left colic, inferior mesenteric artery) [6, 7]. Postoperative pathological examination is currently the gold standard for evaluating regional lymph node metastasis.

Despite improvements in preoperative imaging, such as computed tomography (CT), magnetic resonance tomography (MRI), and fluorodeoxyglucose positron emission tomography (FDG-PET), it is still difficult to accurately assess nodal involvement. Techniques for intraoperative diagnosis of lymph node metastases, including sentinel lymph node biopsy, also remain to be well established [3]. If lymph node metastases were able to be accurately diagnosed at the time of surgical retrieval, the extent of lymph node dissection could be determined in accordance with the nodal status of each patient. Most surgeons believe that appropriate lymphadenectomy can improve short- and long-term outcomes, although evidence supporting the therapeutic significance of lymph node dissection is rather limited [6, 8].

Fluorescence diagnosis using 5-aminolevulinic acid (5-ALA) is a promising technique that has been used to evaluate various kinds of malignancies in neurosurgery [9, 10], urology [11, 12], dermatology [13, 14], thoracic surgery [15], gynecology [16], and gastrointestinal surgery [17]. After exogenous administration of 5-ALA, protoporphyrin IX (PPIX), the last metabolite in heme synthesis, accumulates in malignant cells. Although the mechanism of PPIX accumulation has not been completely defined, several factors concerning 5ALA metabolism, such as reduced

activity of ferrochelatase, which converts PPIX to heme, low Fe^{2+} levels, and increased NO, are possibly involved rather than 5ALA uptake [18-20]. PPIX can be detected as red fluorescence when excited by blue light with a wavelength of 405 nm, enabling tumor lesions to be distinguished from normal tissues.

In this study, we evaluated the effectiveness of fluorescence diagnosis, using 5-ALA administered intraperitoneally or orally, for the detection of lymph node metastases from colon cancer in an experimental setting.

Materials and methods

Animals

5- to 8-week-old female KSN nu/nu mice were purchased from Japan SLC (Hamamatsu, Japan). The mice had been raised from birth in a specific pathogen-free environment, and all in vivo experiments were conducted in accordance with the institutional guidelines of Kyoto University Hospital.

Cell line

Wild-type and green fluorescent protein (GFP)-labeled human colon adenocarcinoma cell line, HCA7, was cultured in D-MEM (Wako Pure Chemical Industries, Osaka, Japan) with 10% serum and penicillin/streptomycin and maintained at 37°C in a humidified incubator with 5% CO_2 in air. HCA7 cell line stably expressing GFP was generated by retroviral transfection with GFP, as described previously [21].

Tumor model

An orthotopic colon cancer model was prepared by inoculating the cecal wall of the mice with cancer cells. The animals were anesthetized with diethyl ether and then underwent laparotomy via a midline incision. After the cecum was exposed, 1×10^6

tumor cells in 30 μ l of sterile PBS were injected into the subserosal layer of the cecal wall with a 30-gauge needle. The abdominal wall was closed with a 5-0 surgical suture in one layer. After tumor inoculation, the animals were kept under standard laboratory conditions: food and water were available *ad libitum*, and illumination was provided according to a 12-hour light/dark cycle.

5-ALA administration

5-ALA (5-aminolevulinic acid hydrochloride, Cosmo Bio, Tokyo, Japan) was diluted to 10 mg/ml with distilled water just before use. Three weeks after surgery, 40 mg/kg of 5-ALA was administered intraperitoneally (IP group) or orally (PO group). The animals were kept in a dark room until observation.

Observation

3 or 6 hours after 5-ALA administration, the animals were anesthetized and underwent laparotomy using the prior incision in a darkroom. Fluorescence diagnosis was performed with a 4-mm endoscope (Hopkins II, 30°, Karl Storz GmbH, Tuttlingen, Germany) connected to a CCD camera (TRICAM PDD, Karl Storz) and a D-Light System (Karl Storz). This instrument allows three different modes: conventional white light mode, auto-fluorescence mode, and photodynamic diagnosis (PDD) mode for PPIX fluorescence. These modes can be easily switched through the use of a footswitch or a button on the camera head. In PDD mode, a specific blue light (380-440 nm) is emitted, and the observation filter in the endoscope eyepiece blocks most of the excitation light. A defined amount of blue light passing through the filter allows color contrast and orientation in fluorescence-negative areas. Thus, this mode is highly specialized for the detection of PPIX fluorescence.

After exploring the abdominal cavity with the D-Light System in both white light and PDD modes, we extirpated the intestine along with the mesentery and

examined the mesenteric lymph nodes again in both modes.

Histopathology

After observation, lymph nodes and some other tissues were extracted and soaked in 4% paraformaldehyde for 24 hours. Hematoxylin and eosin (H&E) staining was performed after fixation.

Results

Formation of lymph node metastasis

We treated 23 animals in all, and details of experiments were described in Table 1. Three weeks after cancer-cell inoculation, tumors were successfully generated at the injection site in all experiments (Fig. 1a). We identified metastases in some meso-colonic lymph nodes (Fig. 1b). Large numbers of small nodules spreading along the mesenteric vessels were found in all 23 mice (Fig. 1a). These nodules were ascertained to be metastases from cecal tumors on both histopathological examination and GFP fluorescence (Fig. 1c). In addition, abdominal wall implantations and peritoneal metastases had developed, accompanied by the accumulation of more or less bloody ascitic fluid (Fig. 1a).

IP group (5-ALA injected intraperitoneally)

Typical PPIX red fluorescence was observed in the skin, gallbladder, and small and large intestines after intraperitoneal injection of 5-ALA (data not shown). In 3 of 10 animals, meso-colonic lymph nodes showed red fluorescence focally and were pathologically confirmed to be metastatic (Fig. 2a, 2b, Table 1). In contrast, non-metastatic lymph nodes were fluorescence-negative (Fig. 2a, 2b). All nodules in the mesentery that were visible under white light showed clear red fluorescence (Fig. 3a, Table 1). Primary tumors and abdominal wall implantations also showed red

fluorescence (Fig. 3b, 3c). Moreover, PPIX fluorescence was detected in peritoneal tissues around the stomach, which proved to be metastases to the pancreas (Fig. 3d). The intensity of the fluorescence at 3 hours was considerably stronger than that at 6 hours.

PO group (5-ALA given orally)

Red fluorescence in the skin, gallbladder, and small and large intestines was observed after oral administration of 5-ALA, similar to that in the IP group (data not shown). PPIX fluorescence was not detected in any meso-colonic lymph nodes or mesenteric nodules at either interval (Fig. 4, Table 1). Only faint fluorescence was seen in some parts of primary tumors and abdominal wall implantations (data not shown).

Discussion

Fluorescence diagnosis using 5-ALA has been studied in various fields since Marik et al. first reported the value of this photosensitizer in 1987 [22]. Neurosurgeons in Europe have already used this technique clinically for the intraoperative detection of malignant gliomas [10, 23]. The European Association of Urology guidelines also recommend photodynamic diagnosis using hexaminolevulinate (HAL), a hexyl ester derivative of 5-ALA, for the detection of bladder carcinomas [24]. Although the mechanisms of PPIX accumulation remain to be fully defined, this phenomenon is thought to be very tumor-specific. To date, few studies have focused on the ability of 5-ALA to detect lymph node metastases from cancers [25-29]. Thus, we evaluated the usefulness of fluorescence diagnosis using 5-ALA for the detection of lymph node metastases from colon cancers. Our results demonstrated that PPIX fluorescence induced by intraperitoneal administration of 5-ALA, not by oral, allowed metastatic lymph nodes to be accurately diagnosed in this mouse model.

Before conducting in vivo experiments, 5-ALA-mediated PPIX accumulation

in HCA7 was confirmed by fluorescence microscopy, using an in vitro method similar to that employed in previous studies (data not shown) [25, 30]. To induce regional lymph node metastases, we orthotopically injected a suspension of the HCA7 cell line. The metastasis rate of this model was estimated at approximately 25% by earlier experiments. In addition, a substantial number of nodules in the mesentery were formed in all 23 mice within 3 weeks. Histopathological examination confirmed that most of these nodules contained cancer cells and were thus considered metastases from the cecal tumor. Because the nodules were distributed along the mesenteric vessels and appeared to be located between the peritoneum, they were most likely metastatic lymph nodes rather than peritoneal dissemination.

Our results in the IP group showed that 5-ALA-induced PPIX fluorescence was successfully detected in metastatic portions of regional lymph nodes, while no apparent fluorescence was observed in non-metastatic nodes. Actually, the distribution of fluorescence was associated with pathological findings. We detected PPIX fluorescence in meso-colonic nodes in 3 of 10 animals, and these fluorescent nodes were pathologically confirmed to be metastatic. This ratio would be compatible with the metastatic rate of this model. Unfortunately, we had performed pathological examination not for all lymph nodes, so we cannot exactly describe the sensitivity or specificity of this technique. However, mesenteric nodules, which were formed in all animals and detected macroscopically, showed clear red fluorescence. The fluorescence was bright enough to detect tiny tumors, which were hardly visible under white light. These results suggest the strong ability of intraperitoneal 5ALA to detect cancer tissues. In addition, primary tumors, abdominal implantations, and pancreatic metastases also showed red fluorescence, suggesting that intraperitoneal 5-ALA might be applied for the intraoperative detection not only of lymph node metastases but also of other occult peritoneal metastases.

Fluorescence at 3 hours was much stronger than that at 6 hours in the IP group.

A previous study using colon carcinoma cell lines for a peritoneal carcinosis model reported that PPIX accumulation and fluorescence peaked 2 to 4 hours after intraperitoneal lavage with 5-ALA [31]. For bladder carcinomas, it is also recommended that 5-ALA is administered intravesically 2-3 hours before cystoscopy. On the other hand, Sroka et al. demonstrated that the maximum contrast of fluorescence intensity between tumors and non-malignant organs was observed 4 to 6 hours after intravenous injection of 5-ALA in a subcutaneous tumor model with colon adenocarcinoma cells [32]. The time to the peak fluorescence may differ depending on the route of administration. Our results suggest that 2 to 4 hours after intraperitoneal 5-ALA administration is the optimal time for detecting lymph node metastases from colon cancers.

Our results indicated that PPIX fluorescence could not be detected in metastatic lymph nodes at 3 or 6 hours after oral administration of 40 mg/kg of 5-ALA. We used the same dose as that in the IP group, and the fluorescence intensity of the skin, gallbladder, and intestines appeared to be similar in both groups, suggesting no appreciable difference in systemic distribution of 5-ALA. The PPIX concentration does not significantly differ between oral and intravenous administration, because oral 5-ALA is well absorbed in the upper part of the intestines [23, 35, 36]. Gahlen et al. compared systemic with local administration of 5-ALA for the fluorescence diagnosis of intraperitoneal tumors [33]. They found that local administration was superior; nevertheless, the total dose of 5-ALA was 4- to 5- fold higher than that used for intravenous injection. One hypothesis proposes that intraperitoneally administered 5-ALA is absorbed through the peritoneum and distributed via both the systemic circulation and by direct uptake into peritoneal tissues, resulting in higher 5-ALA concentrations and greater PPIX accumulation in lymph nodes and other intraperitoneal organs as compared with systemic administration [31, 33, 34].

A recent study demonstrated that intravenous injection of 5-ALA was extremely sensitive for the detection of lymph node metastases from rectal cancers,

even though the dose was as high as 250 mg/kg body weight [25]. A study in breast cancer reported that it was rather difficult to detect the fluorescence intraoperatively in metastatic sentinel lymph nodes after oral administration of 30 mg/kg of 5-ALA, although the fluorescence intensity of metastatic lymph nodes was significantly higher than that of non-metastatic lymph nodes [27]. Another study showed that the porphyrin concentrations of cancerous tissues rose dose-dependently after an intravenous dose of up to 300 mg/kg of 5-ALA [37]. These findings suggest that PPIX fluorescence would probably be detected after oral administration of a much higher dose of 5-ALA than that in our model. Many experimental studies have indicated that 5-ALA can be administered in much higher doses without serious side effects [23, 38]. Nonetheless, most clinical studies have used relatively low doses of up to 60 mg/kg body weight [17, 26-28, 34, 39], and the only approved dose of oral 5-ALA is as little as 20 mg/kg for the detection of gliomas [23, 38]. Increasing the oral dose of 5-ALA thus appears to be impractical for the fluorescence diagnosis of lymph node metastases.

In this small study, we had not fully evaluated the sensitivity and specificity of 5-ALA-induced fluorescence diagnosis of lymph node metastasis. Further precise studies with this animal model which verify the sensitivity and specificity are required to promote clinical translation of this technique.

The penetration depth of red light (630 nm) ranges from 0.2 to 2 cm, and that of exciting blue light (380-440 nm) is even shallower [31]. Moreover, the penetration depth of intraperitoneally administered 5-ALA within lymph nodes is unknown. As discussed previously, these factors might affect the detecting ability in deeper tissue layers [25, 31]. However, the marginal sinus is generally regarded as the initial site of cancer-cell proliferation in regional lymph nodes [40]. Furthermore, PPIX fluorescence induced by intraperitoneal 5-ALA was strong enough to detect, easily and effectively, small metastases within 1-2 mm in diameter in our mouse model. Diagnostic capability for human cancers associated with large lymph nodes, thick layers of endoceliac fat, or

both should be evaluated in future.

There is another limitation in translating our technique to clinical use. Intraperitoneal administration of 5-ALA should be performed under general anesthesia, but it may not be practical to wait for more than 2 hours in the operating room while avoiding exposure to light. Derivatives of 5-ALA, such as HAL, which have better bioavailability than the parent molecule, might solve this problem by shortening the incubation time [41]. Such derivatives are also expected to more potently induce fluorescence in cancers after intraperitoneal administration [42].

In conclusion, our results demonstrate that fluorescence diagnosis using intraperitoneally administered 5-ALA efficiently detects lymph node metastases from colon cancer in mice. With further investigations, this technique would serve the intraoperative diagnosis of lymph node metastases in patients with colon cancer, and real-time fluorescence-based lymph node dissection may become possible.

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Figure captions

Figure 1. Formation of lymph node metastasis 3 weeks after orthotopic inoculation of HCA7.

(a) Macroscopic imaging of the abdominal cavity. Metastases in meso-colonic lymph nodes (arrow heads) were identified in approximately 25% of the mice. The primary tumor (T) and abdominal wall implantations (*) were also developed. In addition, large numbers of nodules were formed in the mesentery (arrows). (b) White light image of a meso-colonic lymph node metastasis (arrow head). (c) Mesenteric nodules that were visible under white light showed GFP fluorescence under PDD mode (right panel) in the experiments using GFP-labeled cells.

Figure 2. Fluorescence imaging of metastatic lymph nodes after intraperitoneal injection of 5-ALA (IP group).

(a) PPIX fluorescence was detected in metastatic parts of meso-colonic lymph nodes (arrow). Non-metastatic lymph nodes were fluorescence-negative (arrow heads). (b) H&E sections of the lymph nodes shown in a; corresponding to the arrow (right panel) and the arrow head (left panel). (Magnifications: 40×)

Figure 3. Fluorescence imaging of other cancerous lesions after intraperitoneal injection of 5-ALA (IP group).

(a) All nodules in the mesentery that were visible under white light showed clear red fluorescence under PDD mode. Non-metastatic meso-colonic lymph nodes were fluorescence-negative (arrow heads). (b, c) The primary tumor and abdominal wall implantation showed red fluorescence. (d) PPIX fluorescence was also detected in metastases to the pancreas. (Left panels; white light mode, right panels; PDD mode)

Figure 4. Fluorescence imaging after oral administration of 5-ALA (PO group).

PPIX fluorescence was not detected in any meso-colonic lymph nodes (arrow) or mesenteric nodules (arrow heads) at either interval. (left panel; white light mode, right panel; PDD mode).

Figure 1

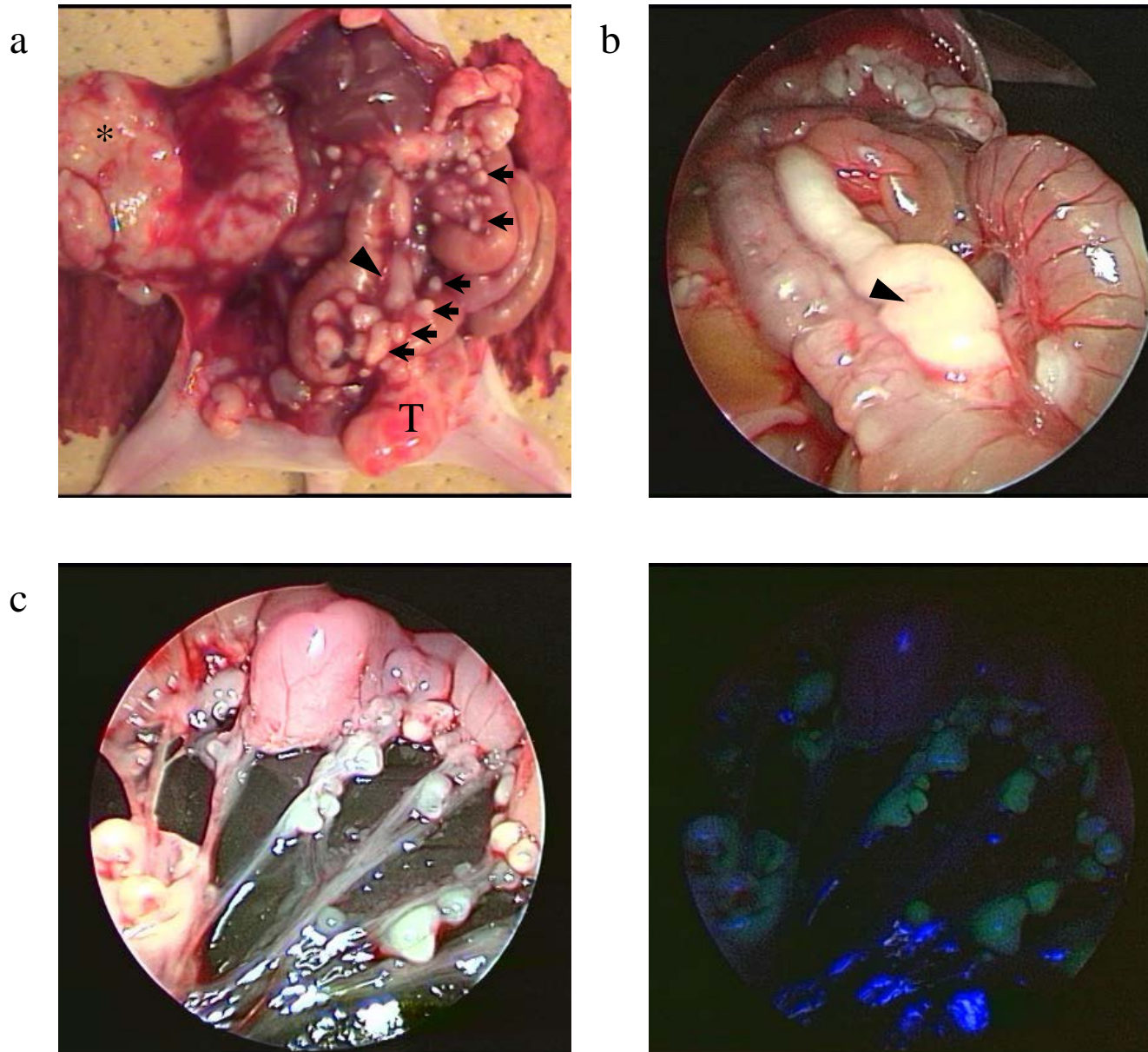
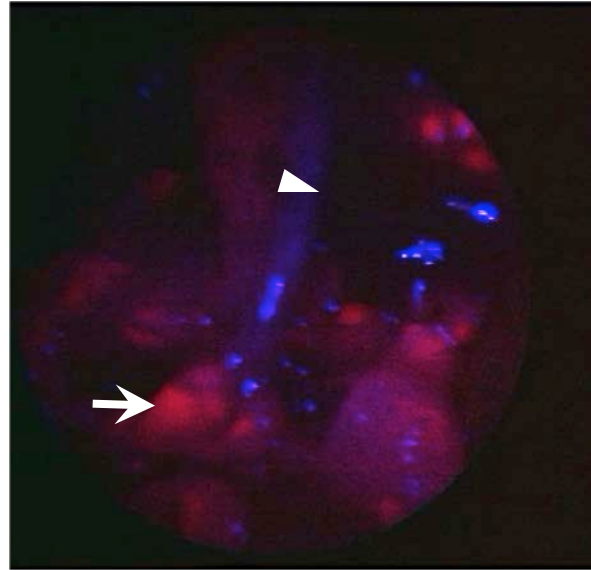
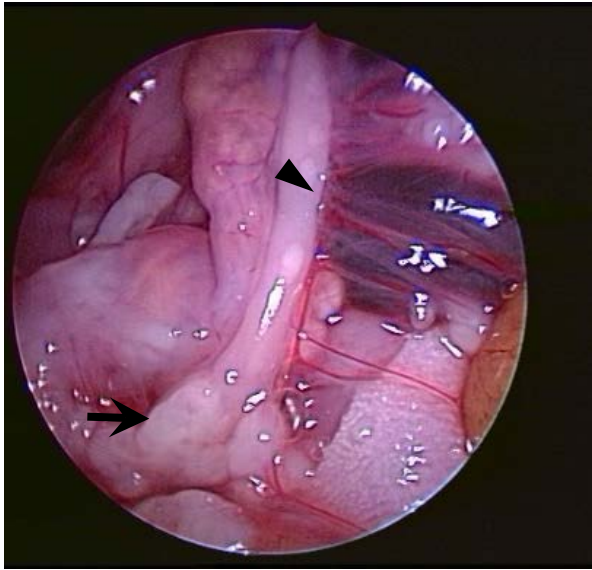


Figure 2

a



b

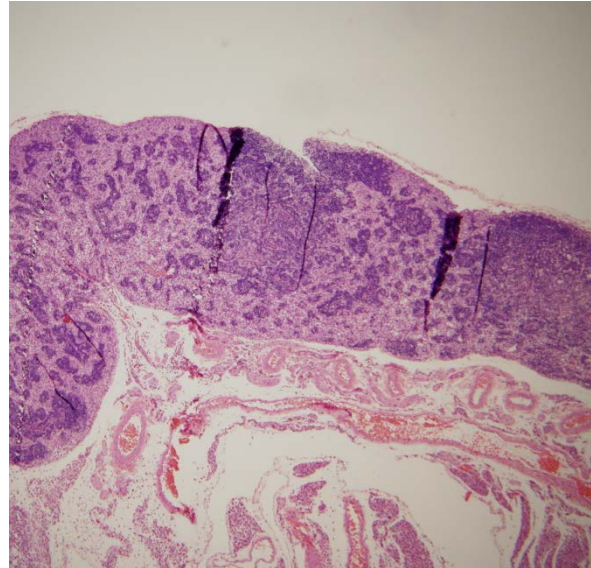
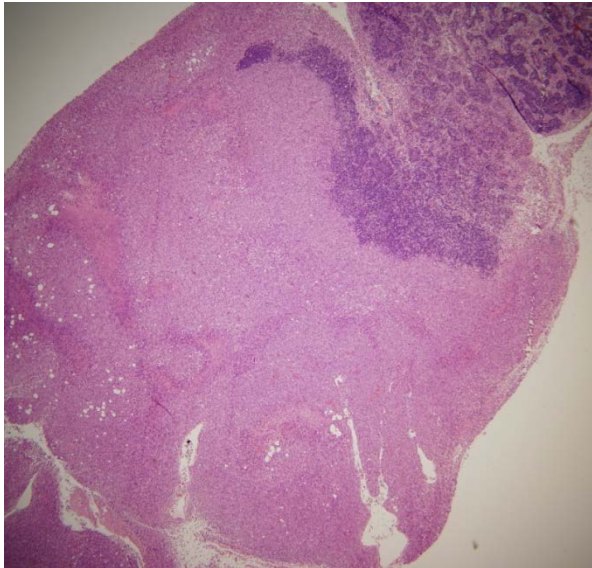
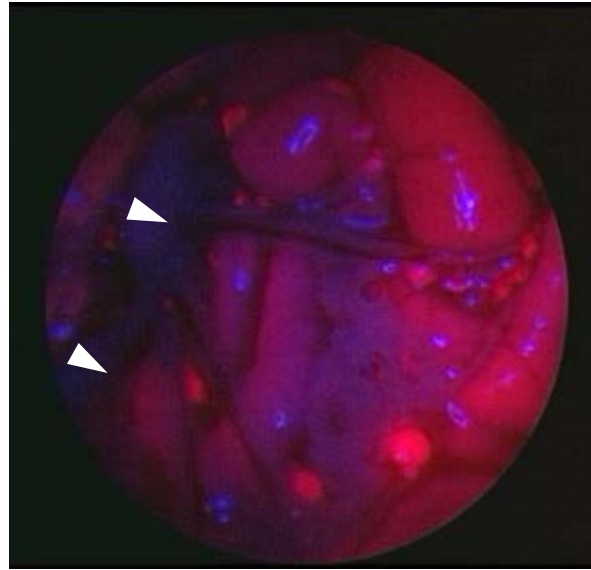
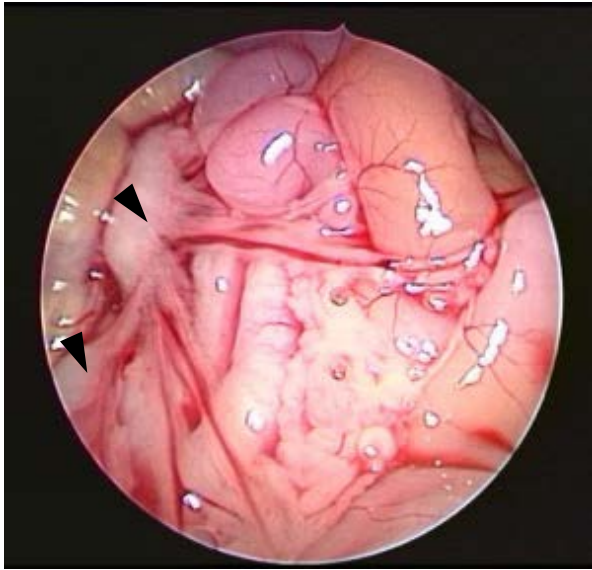


Figure 3

a



b

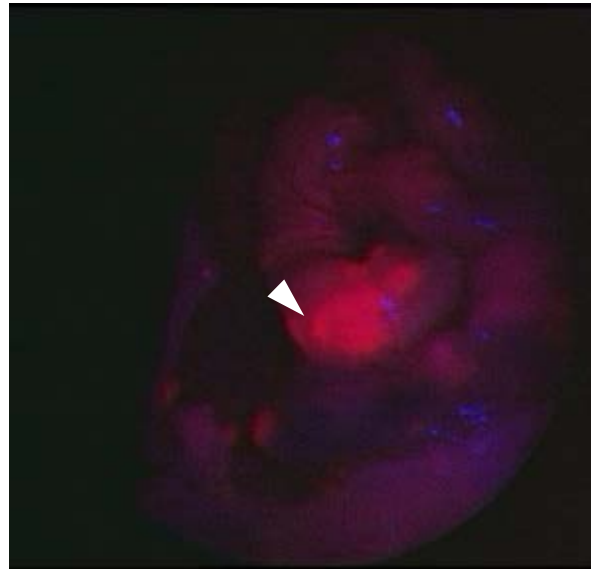
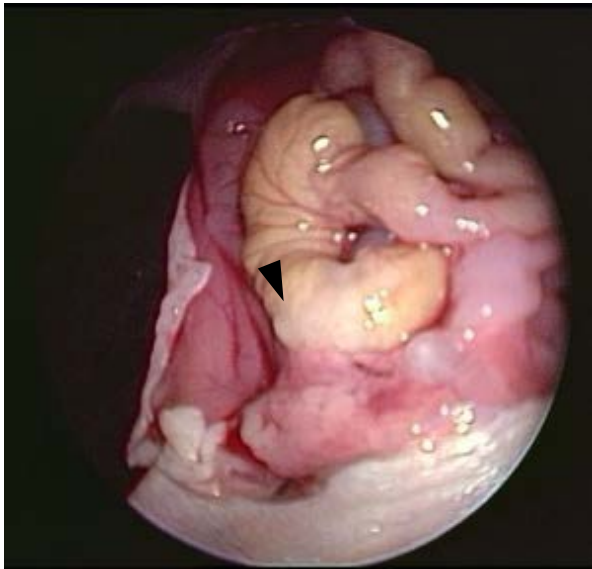
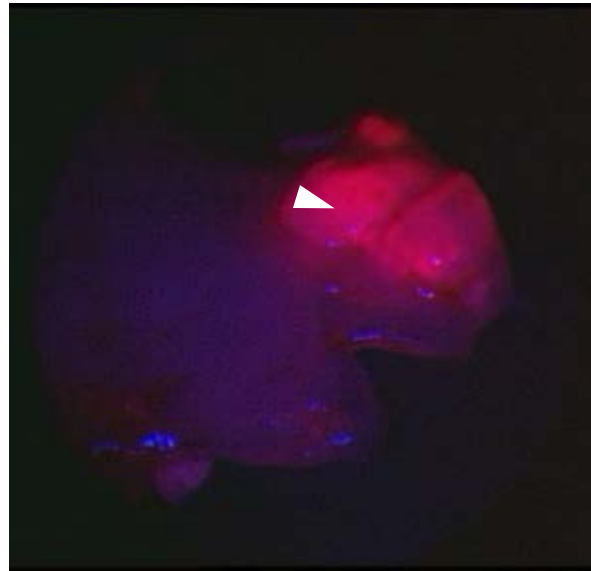
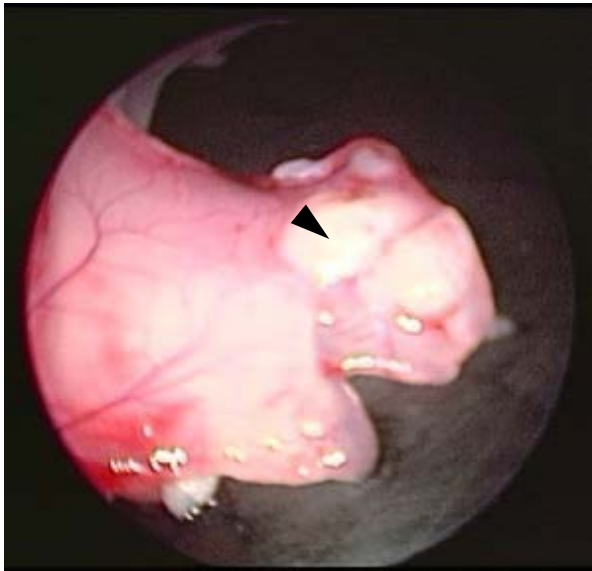


Figure 3

c



d

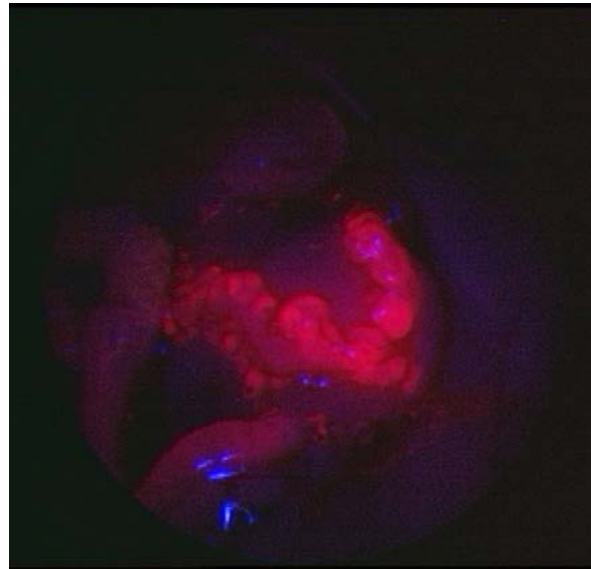
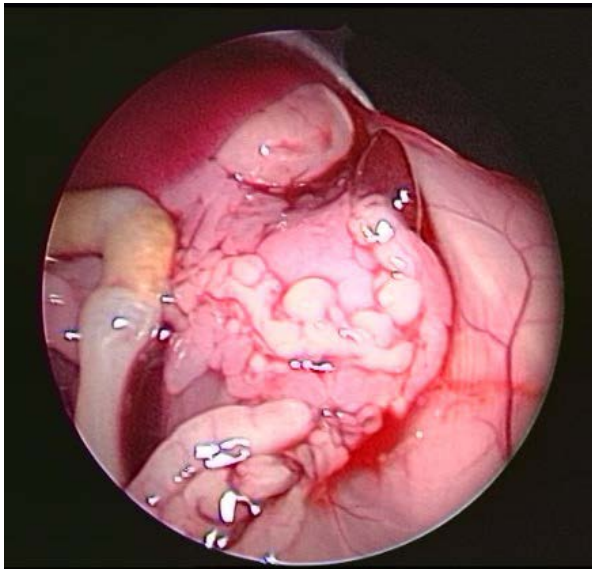


Figure 4

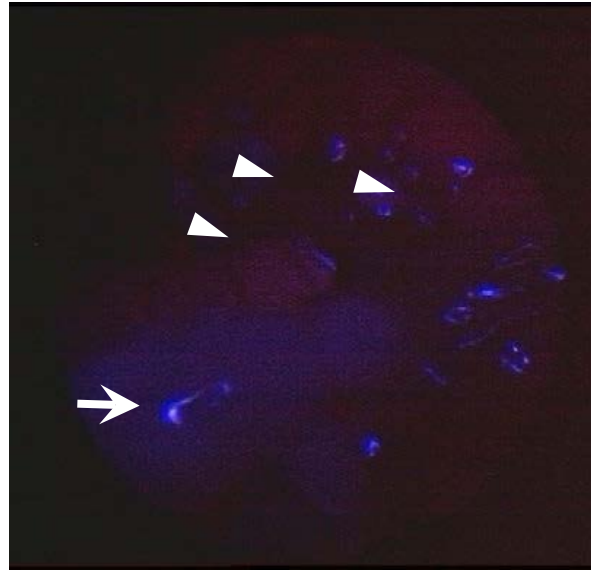
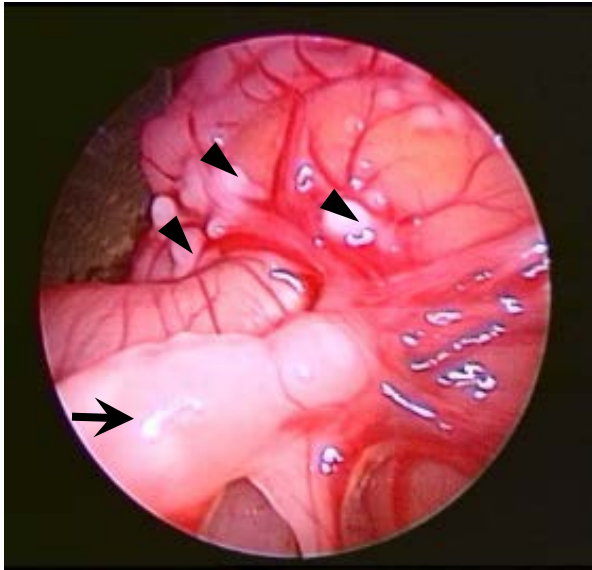


Table 1. Details of Experiments

Route of administration	IP		PO	
Time after administration	3hrs	6hrs	3hrs	6hrs
No. of animals	4	6	3	10
PPIX fluorescence in meso-colonic nodes, No.				
Positive	1	2	0	0
Negative	3	4	0	0
PPIX fluorescence in mesenteric nodules, No.				
Positive	4	6	0	0
Negative	0	0	3	10